E.W. Bolle-Jones: Nutrition of Hevea Brasiliensis I. Experimental Methods

THE

JOURNAL

OF THE

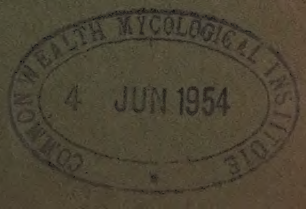
RUBBER RESEARCH INSTITUTE

OF

MALAYA

MAY 1954

Vol. 14 Communication 289



Correct abbreviation for citing references, according to the World List of Scientific Periodicals:
J. Rubb. Res. Inst. Malaya

NUTRITION OF HEVEA BRASILIENSIS
I. EXPERIMENTAL METHODS

By

E.W. BOLLE-JONES

A sand culture technique suitable for the production of Hevea plants of known nutritional status, and analytical methods for the estimation of the macro and micro nutrients and chlorophyll in the laminae have been investigated and satisfactory procedures established.

New, rapid and simple methods are described for the estimation of petiolar rubber content and the chromatographic determination of each sugar present in the laminae.

As a preliminary to the detailed investigation of the mineral nutrition of *Hevea brasiliensis* it was desirable to develop suitable culture techniques and methods of assay for mineral, chlorophyll, carbohydrate and rubber contents of the plants. It was frequently found necessary to modify recognised procedures and the following is an account of these modifications and techniques subsequently used in nutritional studies which will be reported later.

GROWTH OF HEVEA BRASILIENSIS IN SAND CULTURE

Five-gallon clay pots painted on the inside with three coats of bituros paint (HEWITT 1952) were found satisfactory for the growth of plants up to the age of ten months. The basal, 1 inch, drainage hole was covered with a thin weft of acid-extracted glass wool on which was placed an inverted watch glass. This type of container was found satisfactory for the investigation of major nutrient and iron deficiency symptoms. Five-gallon glass demijohn jars, which had the neck regions cut off and $\frac{1}{4}$ inch drainage holes drilled in the base, were also used successfully to study the effects of iron and manganese deficiency. These containers had the advantage of being obtained cheaply and could be scrubbed or steamed out between experiments and required no painting.

Sand samples taken from different areas were water washed, oven dried and extracted with constant boiling hydrochloric acid and the extracts analysed. The results (TABLE I) indicated that river sand from the R.R.I. Experiment Station Sungei Buloh, generally contained less magnesium, calcium, and iron than the other samples but that the potassium content was relatively high. On this evidence the Experiment Station sand was chosen for use in the pot culture experiments. Later analyses, conducted on different batches of sand taken from the same river area over a

TABLE 1: MINERAL CONTENT (SOLUBLE IN HYDROCHLORIC ACID) OF DIFFERENT SANDS

(expressed as parts per million of dry sand)

Sand location		*Colour*	*Mg*	*K*	*Ca*	*Fe*	*Mn*	*Cu*	*Zn*
Port Dickson	*White House*	*White*	101	7.4	878	3,180	5.8	1.6	7.5
Port Dickson	*Drumochter*	*Pale fawn*	62	7.3	90	2,500	10.4	0.3	5.9
Port Dickson	*Drumochter*	*Grey*	90	6.5	182	45	1.8	0.2	4.5
Port Dickson	*6th Mile*	*White interdispersed with brown grit*	56	17.7	61	2,770	6.6	0.7	10.5
Port Dickson	*South Bay*	*Brown*	280	7.6	45	21,000	25.5	0.7	11.5
Port Dickson	*8th Mile*	*Brown*	201	18.8	121	11,250	11.9	3.0	11.0
Morib	*Bungalow*	*White*	117	43.2	65	460	12.3	0.2	2.3
Morib	*Batu Laut*	*White*	95	55.4	75	400	13.6	0.5	3.0
Cheras	*9th Mile*	*Whitish brown*	4	34.9	41	780	9.4	0.3	2.5
Cheras	*Left bank*	*Pale brown*	375	132.0	68	2,320	60.6	0.4	8.0
Cheras	*Right bank*	*Pale brown*	228	7.4	61	1,650	58.9	0.2	4.0
Expt Station	*Field 37*	*Light brown*	10	16.8	11	130	6.7	0.3	2.5

The upper part of the TABLE concerns sea sands, while the lower (beneath the rule) concerns river sands.

period of time, indicated that the mineral content showed considerable variation which presumably was caused by variable local conditions at the time of collection such as depth of digging, heavy rainfalls and other factors. Fractionation of the sand according to particle size, with the rejection of particles larger than 1.5 mm, showed (TABLE II) that the concentrations of magnesium and potassium in each fraction increased as the particle size grew smaller whereas the reverse was true for calcium, iron and manganese.

It was decided to use sand between 0.5 – 1.5 mm particle size for the pot experiments. This fraction had the advantage of containing less magnesium and potassium than the fine fraction (0 – 0.5 mm) and a decreased proportion of calcium, iron and manganese as compared with the coarse fraction (1.0 – 1.5 mm). It was found to give free drainage and had a suitable water retaining capacity; thus plants given 1 litre of nutrient daily showed no signs of wilting.

TABLE II: MINERAL CONTENT (SOLUBLE IN HYDROCHLORIC ACID) OF GRADED EXPERIMENT STATION SAND

(Means of results obtained with different sand batches.)

Particle Size (mm)	*Dry sand (p. p. m.)*							
	P	*Mg*	*K*	*Ca*	*Fe*	*Mn*	*Cu*	*Zn*
1.50 — 1.00	6.3	5.7	8.2	10.5	1202	97.5	0.6	1.5
1.00 — 0.50	8.3	9.5	12.1	2.5	953	27.9	2.3	4.6
0.50 — 0.00	8.6	11.8	15.5	4.5	484	17.0	2.9	3.1

To ensure consistent results and to avoid large variation in available nutrient content of different batches the sand fraction separated for use in pot cultures was allowed to soak in cold 2% hydrochloric acid (Wt HCl/tot. vol.), with frequent agitation for three days. The acid was drained rapidly, the black residual scum scraped off the surface of the sand which was then vigorously leached with tapwater before transference to the pots. Leaching of sand in the pots was continued with calcium nitrate (32 milligram equivalents Ca^{++}/litre) until the leachate was blue to bromocresol green. Sand purified in this manner was used in the investigation of macronutrient deficiencies.

When sand was required for iron, manganese and boron deficiency investigations, the product obtained from the 'cold' acid soaking and subsequent tapwater washing was transferred to a glazed porcelain vat and saturated with 10% hydrochloric acid. Steam was then passed into the sand-acid mixture for twelve hours; the sand was subsequently leached rapidly with tapwater and transferred to pots where it was leached with calcium nitrate, purified relative to each micronutrient.

Distilled water was used for watering the iron, manganese and boron deficient plants and tapwater for the macronutrient deficiency studies. The composition of the tap water (p.p.m.: P = 0.02, Mg = 0.18, K = 1.8, Ca = 1.2, Fe = 0.16, Mn = 0.01) did not prevent the appearance of severe nitrogen, sulphur, phosphorus and magnesium deficiency symptoms but it later became necessary to use distilled water for the satisfactory production of symptoms in potassium and calcium deficient plants.

Nutrient concentrations suitable for temperate crops (HEWITT 1952) were not satisfactory for *Hevea brasiliensis*. It was found necessary to reduce the phosphorus and calcium to relatively low concentrations and to increase that of iron to avoid the occurrence of iron deficiency chlorosis. The composition of the nutrient which hitherto has given the most flourishing growth is, in milligram equivalents per litre, NO_3^- 7.0, SO_4^{--}4.0, PO_4^{---}3.0, Mg^{++} 2.0, K^+ 4.0, Ca^{++}4.0, Fe^{+++} 0.66 – 1.00. The micronutrients were applied for the earlier experiments in the same concentrations as prescribed by HEWITT (1952 page 189) but later the boron concentration was reduced or even omitted as it was found that the plants absorbed and accumulated extremely large amounts of boron.

The efficiency of these culture techniques in general, can be judged from *Figures* 1 to 5 and TABLE III. Clear and marked retardation of growth as a result of mineral deficiency was obvious for all treatments at the five month stage except for the boron and potassium deficient plants. Recovery was attributed in the boron deficient plants to a poor nutrient purification procedure and to the suspected presence of boron in the sand. Recovery in the potassium deficient plants was probably due to the high concentration of potassium in the sand. Later, the effects of potassium deficiency (*Figures* 3, 4,) became marked and severe. It was noticeable visually and apparent from TABLE III that the plants grown in the clay pots were more vigorous and heavier than those grown in glass pots thus indicating that the roots were able to extract more nutrient from the walls of the clay than the glass pots.

TABLE III: TOTAL WEIGHT OF SHOOTS AND ROOTS PER PLANT (IN GRAMS) FIVE MONTHS AFTER SOWING

Tjir 1 seedlings

Treatment	*Dry weight*	*Treatment*	*Dry weight*	*Treatment*	*Fresh weight*	
					Glass pot	*Clay pot*
Complete	30.7	*-K*	21.0	*Complete*	76	—
-N	8.2	*-Cu*	15.7	*-Fe*	44	58
-S	12.8	*-Fe*	16.7	*-Mn*	35	60
-P	14.0	*-Mn*	16.7	*-B*	74	87
-Mg	6.9	*-B*	28.0			

Min. 5% sig. dif. = 7.2

Min. 1% sig. dif. = 10.3

Figure 1. Three month old Tjir 1 seedlings. Complete rutrient plants (left) magnesium deficient plants (right). Note interveinal chlorosis appearing near midrib of midstem laminae of magnesium deficient plants which were weaker and fewer leaved.

Figure 2. Six month old Tjir 1 seedlings. Complete nutrient plants (left) iron deficient plants (right). Note acute iron deficiency causing marked defoliation, death of growing point (left hand plant) and chlorotic young laminae (right hand plant).

Figure 3. Seven month old Tjir seedlings. Potassium deficient plants (left) complete nutrient plants (right). Note general paleness of potassium deficient plants due to chlorosis of midstem laminae.

Figure 4. Close up of potassium deficient leaflets. Note marginal scorch which follows chlorotic mottling.

Figure 5. Ten month old Tjir seedlings. Phosphorus deficient plants (left) complete nutrient plants (right). Note stunting, thin stem and small sized leaves of phosphorus deficient plants.

DETERMINATION OF THE MINERAL CONTENT OF THE PLANTS AND DETECTION OF CONTAMINATION IN WATER SUPPLIES

CHEMICAL METHODS

Leaf material when dried and powdered was wet digested with nitric and perchloric acids and the final digest used for the analysis of sulphur, phosphorus, magnesium, potassium, calcium, and manganese. Nitrogen, iron, copper, zinc and boron were determined independently on separate quantities of leaf material. Mention will only be made here of recent investigations in this laboratory on the reliability of methods of analysis as applied to rubber leaves.

Satisfactory methods which could be used for the direct analysis of the wet digest aliquots were available for the estimation of sulphur (gravimetric—as barium sulphate), phosphorus (colorimetric—as the reduced phosphomolybdate blue complex) and calcium (titrimetric—oxalate precipitation and subsequent permanganate titration).

HUNTER'S method (1950) of magnesium estimation, (based on the formation of a coloured complex of magnesium hydroxide with Clayton yellow dye) was investigated and found to be fully satisfactory. The method covered a range of 0-0.14 mg of magnesium; recovery values did not vary more than 10% from the amount added. The method was more sensitive than the one previously operated (DROSDOFF and NEARPASS 1948).

A method of potassium estimation previously used by the author and based on unpublished procedures developed by FORSTER (1949) proved to be suitable in the limited range of 0.1-0.4 mg of potassium. The method, which demanded careful manipulation, depended on the precipitation of potassium as the cobaltinitrite which was then separated, decomposed by the addition of weak nitric acid and thiocyanate added. The intensity of the resultant blue colour given by the cobalt-thiocyanate complex was measured absorptiometrically. The recovery values obtained from the addition of known amounts of potassium to plant material during the course of digestion ranged from 100-111%. Care was taken to digest the potassium standards in the same manner as the leaf material and to exclude sulphuric acid from the digest. This colorimetric method required the equivalent of 10-20 mg of dried lamina for the estimation whereas the volumetric method, which was based on the oxidation of potassium cobaltinitrite with permanganate required 250-500 mg of material. Thus the method was suitable for nutritional studies where leaf material was limited; the volumetric method was otherwise preferred as it was less exacting to operate and gave consistently reproducible results.

CORNFIELD and POLLARD (1950) described a method of estimating microquantities (0.1-1.0 μg) of manganese using the tetramethyldiaminodimethylmethane reagent and a dry ashing procedure. It was found that this method could be applied directly to aliquots of wet digests. At first phenolphthalein was used to

estimate the neutralisation point of the wet digest aliquot but this indicator was later found to interfere with the method and its addition was deleted without any ill effects on the recovery value. No further neutralisation beyond the addition of the acetate-acetic reagent prescribed by Cornfield and Pollard was necessary. As for potassium consistently good recoveries could only be obtained when each manganese standard was digested in the same manner as the leaf material. The recovery values obtained when known amounts of manganese were added to leaf material before digestion ranged from 90 to 110%. The method of estimating the permanganate formed from the oxidation of manganese present in the aliquot (WILLARD and GREATHOUSE 1917, RICHARDS 1930) gave better recovery values than the 'tetrabase' method but usually required 30-50 times as much aliquot. Extensive comparisons of analytical results obtained by both methods on leaf material showed, in general, that the 'tetrabase' method gave the higher values, but was much the better suited for the investigation of the mineral status of manganese deficient laminae.

The Kjeldahl method hitherto employed for the estimation of leaf nitrogen used selenium as a catalyst in the presence of sulphuric acid. This procedure gave incomplete recoveries (59%) for nitrogen (as nitrate) added to leaf material as also did the following methods: CHIBNALL, REES and WILLIAMS, 1943 (72%) and STREET, KENYON and WATSON, 1946 (53%). To date, the method which gave the most satisfactory recoveries (80%) employed selenium in addition to sodium thiosulphate and salicylic acid, as a catalyst in the Kjeldahl incineration procedure described in the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists (1945) handbook.

Numerous procedures for the determination of iron in leaf material were tried but without consistent results. The most troublesome factors appeared to be the presence of silica which adsorbed the iron during the course of the wet digestion (MASON 1951) and uncontrollable variations in the white 'dry heating' or 'baking' stage of the digestion procedure. A method recently developed by PICKARD (1952) proved superior to all others tested and gave good recovery values, provided rigorous control over the digestion procedure was maintained. Half a gram of lamina was digested with sulphuric, nitric and perchloric acids. Prolonged fuming and complete expulsion of nitric acid fumes from the digest were necessary before the solution was made slightly alkaline by the addition of ammonium citrate. The addition of thioglycollic acid gave a stable reddish purple in the presence of iron. The recovery values obtained for known amounts of iron added to plant material during digestion were good (94-106%) and the calibration curve was perfectly reproducible. The method suffered the disadvantage of requiring a separate digestion of the dry material and stringent precautions against contamination were needed to give reproducible results. Iron values in the range of 0-0.08 mg were determined.

Leaf material (2 gm) to be analysed for copper was digested separately with nitric, perchloric and sulphuric acids. The digest when cooled was diluted with water, boiled, filtered and made up to approximately 70 ml. The solution was brought up to a pH of 8.0-9.0 with ammonia and citric acid and shaken vigorously in the presence of sodium diethyldithiocarbamate according to DRAKE'S method (1950). The copper diethyldithiodicarbamate complex was quantitatively extracted with amyl alcohol and the transmittance measured. Recoveries for 0.02 mg amounts of copper added to 2 gm of leaf material were 105% and 106%; a range of 0-0.08 mg of copper was covered by the method.

Little success was obtained when a monocolour dithizone method (SANDELL 1944) for zinc estimation was applied to wet digested zinc standards. The points on the calibration curve obtained were erratic. A dry ashing procedure described by HEINEN and BENNE (1951) was examined and found more successful. This method gave recovery values of 86%, 99% and 100% for 2.5 μg of zinc when added to 0.125 gm of dry lamina prior to ashing. The method, which depended on the colorimetric measurement of zinc as its dithizonate, operated well over a range of 0-8 μg of zinc.

HATCHER and WILCOX'S (1950) dry ashing method (for the colorimetric estimation of boron as its coloured carmine complex) was used to estimate boron within the range 0-10 μg. Recovery values obtained from the addition of boron to 0.5-1.0 gm of dried lamina varied from 90% to a 100%. Reproducibility of the calibration curve was good and the method was extremely reliable.

BIOASSAY METHODS

The molybdenum contamination of waters, sands and reagents used for pot culture studies was detected and estimated with *Aspergillus niger* Strain M, using the methods described by HEWITT and HALLAS (1951). A variation in molybdenum supplied to the organism from 0-0.02 μg per 50 ml of nutrient solution gave a mycelial weight which provided an effective range of 600 mg (*Figure* 6). This range was less extensive than that reported by HEWITT and HALLAS (1952) but corresponded more closely to that (57-975 mg) described by NICHOLAS (1951). These workers were able to grow their cultures at 25°C whereas the range quoted here was obtained at 28°-29°C. Although mycelial growth was greatly reduced in the 'nil' cultures (100-200 mg) it should have been possible to reduce the 'nil' value to approximately 50 mg.

Rigorous flask cleaning procedures such as hydrochloric acid steaming did not lower appreciably the high 'nil' values. Both macro and micro nutrient salts were heavily contaminated with molybdenum despite successive recrystallisations. It was suspected that in addition to salt contamination the mode of subculturing the fungus on a 'complete' agar medium provided the spores with sufficient molybdenum to fulfil the fungus' growth requirement. Accordingly attempts are being made to subculture

the fungus on successive molybdenum deficient media in order to reduce the molybdenum content of the inoculum and hence increase the sensitivity of the method.

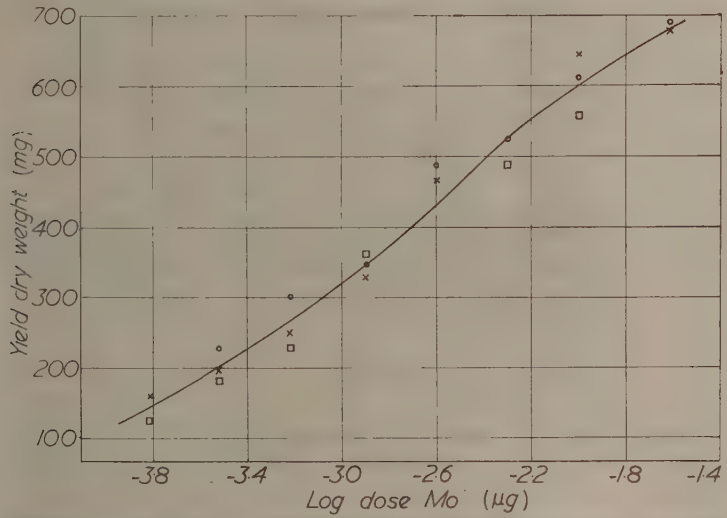


Figure 6. The effect of increasing molybdenum dosage on the mycelial weight of Aspergillus niger Strain M. Each point represents the mean of two cultures.

THE ESTIMATION OF CHLOROPHYLL IN THE LAMINAE OF HEVEA BRASILIENSIS

Chemically pure chlorophyll is difficult to obtain. To prepare a calibration curve for the colorimetric estimation of chlorophyll it was necessary to determine the transmittance, under closely defined conditions, of a chlorophyll solution prepared from *Hevea brasiliensis* laminae and to employ the absorption coefficients determined by COMAR and ZSCHEILE (1942) to calculate the amount of chlorophyll present. The procedures employed were similar to those described by COMAR (1942) and COMAR, BENNE and BUTEYN (1943). 25 gm of fresh laminae were macerated in the presence of 0.5 gm of calcium carbonate with 300 ml of 85% acetone and the final volume made up to 500 ml. A portion (100 ml) of this solution of chlorophyll in acetone was transferred to ether, scrubbed ten times with distilled water in the dark, centrifuged to clarify it and dehydrated by standing in contact with freshly ignited sodium sulphate for 24 hours. Forty ml of this ether solution was made up to 100 ml with dry ether and despatched, stored in ice, to the National Physical Laboratory for spectrophotometric measurement. Per cent transmittance at 6425 and 6600A was determined; using these values and substituting in the following equation of COMAR and ZSCHEILE (1942) it was possible to calculate the concentration

of chlorophyll (as mg/litre) in the sample examined and hence in the original chlorophyll/acetone solution.

$$\text{Total chlorophyll} = 7.12 \log_{10} \frac{I_0}{I_{6600}} + 16.8 \log_{10} \frac{I_0}{I_{6425}}$$

I_0 = intensity of light transmitted by solvent filled cell

I_{6600} = intensity of light transmitted by solution filled cell at 6600A

I_{6425} = intensity of light transmitted by solution filled cell at 6425A

In the meantime aliquots of the original chlorophyll/acetone solution had been taken and made up to 50 ml with 85% acetone. These solutions were 'spekkered' using orange filters. Knowing the strength of the original solution used to prepare the aliquots a calibration curve could therefore be prepared (*Figure* 7).

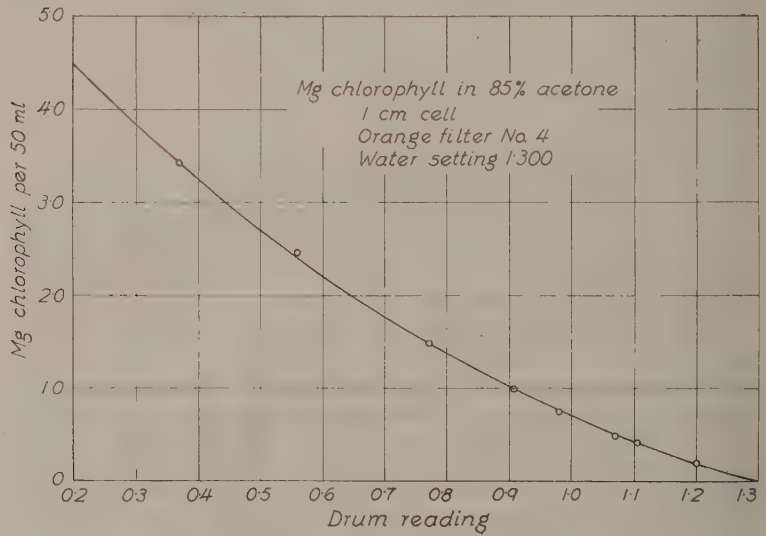


Figure 7. Calibration curve for the estimation of total chlorophyll content of 85% acetone extracts prepared from the laminae of Hevea.

To determine the chlorophyll content of the laminae the following procedure was adopted:

The frozen laminae (1.5 gm) were thawed and macerated with 50 ml of 85% acetone in the presence of 10 mg of calcium carbonate in a stainless steel macerating cup which fitted the 'Ato-Mix' (a bottom drive machine manufactured by the Measuring and Scientific Equipment Ltd). The extraction continued for five minutes. The macerate was filtered, made up to 100 ml with more acetone and then 'spekkered'. The extraction appeared to be quantitative—leaving a perfectly white residue on the filter paper.

THE ESTIMATION OF RUBBER IN PETIOLAR TISSUES

A rapid, but reliable, method was required for the assessment of the rubber content of plants grown in a large number of factorial treatments. It was desired to avoid the use of bromination methods (WILLITS, SWAIN and OGG 1946; MEEKS, CROOK, PARDO and CLARK 1953) owing to the objection of using bromine under tropical conditions for the routine analysis of a large number of samples and also to the possibility of variation of the gravimetric factor for converting weights of bromide to weights of rubber, according to plant source and treatment. The turbidometric methods (TRAUB 1946, RICHES 1948, BONNER and ARREGUIN 1949) were not regarded as being sufficiently reliable when applied as a routine method of analysis. A method developed by SLATTERY and TYSDAL (1950) for the estimation of the rubber content of *Parthenium* laminae was applied to *Hevea brasiliensis*. Tissues were parboiled and macerated with a 1:1 mixture of water and ethyl alcohol. The macerate was poured into a large volume of water and allowed to stand for half an hour at the end of which time a 'worm' of coagulated rubber should have been formed at the bottom of the container. No such accumulation of rubber was found on the several occasions on which the method was tried. This method which was applicable to *Parthenium*, probably because of the latter's higher concentration of rubber in the tissues, was abandoned.

Both SPENCE and CALDWELL (1933) and VAN DER BIE (1946) developed methods which involve a preliminary acetone extraction of the tissue followed by a benzene extraction. Van der Bie's method is reliable but tedious, requires three to four days for the completion of the analysis and, in this laboratory, only a limited number (9) of samples can be conveniently analysed at one time. It was therefore necessary to evolve a more rapid method of determination. The following describes briefly the results of the investigation and the method finally employed for routine determinations.

Petioles were found to yield at least ten times more rubber per unit dry weight than the laminae and were therefore used. Preliminary tests were made using 15 gm of fresh petioles which were frozen in liquid air, coarsely ground in a Wiley mill, transferred to a macerator before thawing out, and extracted with acetone followed by benzene.

The benzene extract obtained from fresh material was generally turbid and after settling the supernatant liquid was still green—indicating an incomplete extraction of pigments by acetone. When these turbid extracts were centrifuged a white granular precipitate of 'rubber' was obtained—usually much less than 100 mg per 15 gm fresh petiole. Centrifuging did not:

(a) precipitate more than 70% (usually far less) of the rubber in the benzene extract, either after the addition of acetone or a mixture of acetone plus alcohol; the precipitate also continued to come down gradually with time even after centrifuging.

(b) give a precipitate which was free of substances hydrolysable by alcoholic potash.

More rubber could generally be extracted from oven dried, milled (40 mesh), petiole material than from the same equivalent fresh weight of frozen, milled (no screen) material. The greater surface area of the dried material exposed to the extracting reagent enhanced the efficiency of the extraction and gave a better agreement between replicates. The acetone maceration extracted very little; it was possible to delete this stage if the strength of the alkali used in the hydrolysis was increased from 0.5 N (as used by VAN DER BIE, 1946) to 1.0 N.

The tentative method which evolved and was used for comparisons with the van der Bie method was as follows:

Fresh petioles were oven dried, at a temperature of 50-70°C for one to two hours, *i.e.* until sufficiently brittle to be milled and passed through a 40 mesh Wiley mill screen. The powdered petiole was again dried and 5 gm weighed out into the bowl of a macerator (Ato-Mix). 100 ml of benzene were added and the mixture macerated for 5 minutes at half speed (6,000 r.p.m.). The extract was filtered through glass wool and the bowl swilled out with another 50 ml of benzene. The benzene extract was gently evaporated to dryness, 100 ml of 1.0 N alcoholic soda added to the residue and the mixture refluxed vigorously for one hour. The supernatant liquid was decanted, the residual film of rubber washed several times with water, followed by 30 ml of 2% formic acid and finally filtered off into a sintered glass crucible, dried for one hour at 105°C, and weighed. This weight was taken as the amount of rubber in the sample. The estimation, beginning at the maceration, can be completed within five hours.

A comparison of some of the results obtained by this procedure with that of van der Bie's method gave a regression line according to the equation $y = 35.74 + 0.6005x$, where b (slope of the regression) = 0.60 ± 0.06, and $a = 35.7 \pm 6.74$ (see *Figure* 8). Thus b differed significantly from unity and a was also significantly different from zero. The values obtained by this method were therefore not directly equivalent to those obtained by van der Bie's procedure but the relationship between both methods was a significant one and comparisons made using the rapid method should agree well with those based on van der Bie's method. No significant difference existed between the results obtained by the two methods for the data represented in *Figure* 8.

A few typical values for the per cent rubber hydrocarbon content (estimated as by BURGER, DONALDSON and BATY 1943) of crude 'rubber' samples obtained by both the accepted van der Bie method and the rapid macerator method together with relevant yield figures are given in TABLE IV. The 'rubber' extracted by the van der Bie method consistently contained a higher percentage of hydrocarbon than that obtained by the macerator method. When the yield of 'rubber' per 5 gm of petiole was multiplied by

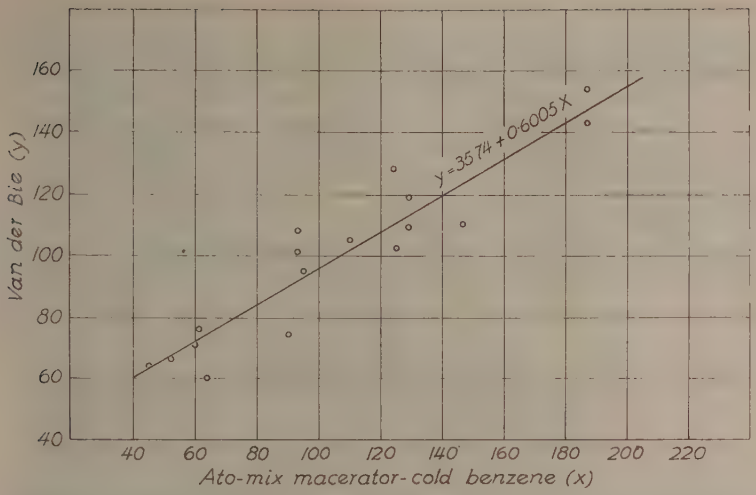


Figure 8. Relationship between amounts of rubber extracted from dried petiolar material by van der Bie's and Ato-Mix macerator (cold benzene) extraction methods. Values given as mg rubber per 5 gm of petiole material.

the respective conversion factor the total amount of rubber hydrocarbon extracted by both methods was found to be in fair agreement (TABLE IV).

TABLE IV: RUBBER HYDROCARBON CONTENT OF RUBBER EXTRACTED BY VAN DER BIE AND THE RAPID MACERATOR METHODS

'Rubber' extracted mg per 5 gm petiole		*Rubber hydrocarbon in 'rubber' %*		*Hydrocarbon extracted mg per 5 gm petiole*	
V der B.	*Mac.*	*V der B.*	*Mac.*	*V der B.*	*Mac.*
74	94	67	49	50	46
72	124	62	42	45	52
96	112	66	51	63	57
88	96	70	60	62	58
67	75	68	54	46	40
117	139	63	53	74	74
113	136	66	52	75	71
74	82	62	53	46	44

The rapid macerator method could also be adapted to the use of smaller weights of dried petiole material than the standard 5 gm. Thus in one experiment extraction of 1 gm yielded 18 mg of rubber; 3 gm yielded 55 mg and 5 gm yielded 91 mg of rubber. These extractions were carried out using the otherwise standardised procedure previously described.

To simplify the procedure further the efficiency of an ordinary mechanical stirrer in the extraction process was examined. Some of these results are presented in TABLE V. It was apparent that warm benzene extracted much more rubber than the cold

TABLE V: EFFICIENCY OF STIRRER AND MACERATOR METHODS IN EXTRACTING RUBBER FROM DRIED PETIOLAR MATERIAL

Time of extraction (min)	*Method*	*Rubber extracted mg per 5 gm petiole*	*Hydrocarbon in 'rubber' %*
5	*Stirrer - Cold benzene*	58	61
5	,, - *Warm benene*	109	52
20	,, - *Cold benzene*	66	60
20	,, - *Warm benzene*	111	60
5	*Ato-Mix macerator Cold benzene*	116	52

Min 5% sig. dif. = 25.2

benzene; this increase was accompanied by a slight decrease in the hydrocarbon content of the rubber for the short period (5 min) of extraction. No difference existed between the long (20 min) and short (5 min) periods of extraction for the weights of rubber obtained using either warm or cold benzene. The warm benzene extraction of 5 minutes compared favourably with the results obtained with the 'Ato-Mix' macerator. Various trial runs were performed using varied times; an arbitary extraction period of 7 minutes was finally chosen. The comparison of hot and cold stirrer extraction, Ato-Mix macerator and van der Bie's methods given in TABLE VI, indicated that the amount of rubber hydrocarbon extracted (*i.e* product of [mg rubber] and [percentage rubber hydrocarbon ÷ 100]) with warm benzene exceeded slightly that of van der Bie's method.

TABLE VI: COMPARISON OF FOUR DIFFERENT METHODS OF RUBBER EXTRACTION FROM DRIED PETIOLAR MATERIAL

Method	*Rubber extracted mg per 5 gm petiole*	*Hydrocarbon in 'rubber' %*
Stirrer - Cold benzene (7 min)	55	64
Stirrer - Warm benzene (7 min)	91	55
Ato-Mix, macerator - Cold benzene (5 min)	82	53
Van der Bie (1946)	74	62

Min 5% sig. dif. = 0.05, = 7.0

Thus the final rapid method, as modified to suit the analysis of a large number of samples became as follows:

5 gm of dried petiole were placed in 250 ml beaker, 100 ml of warm (50-60°C) benzene added and the mixture stirred for

7 minutes at 700 r.p.m. The benzene extract was filtered through glass wool and gently evaporated in black paper covered flasks on a steam bath to a volume of 2-5 ml. The evaporation was completed in an oven at 60-80°C.

30 ml of 1.0 N alcoholic soda were added to the residue and the mixture refluxed for one hour using a Bailey-Walker spiral condenser. The supernatant liquid was decanted and 30 ml of 2% formic acid solution added to the rubber film. The rubber was filtered off into a sintered glass crucible, washed thoroughly with water and 1 to 2 ml of ethyl alcohol, before being dried to a constant weight.

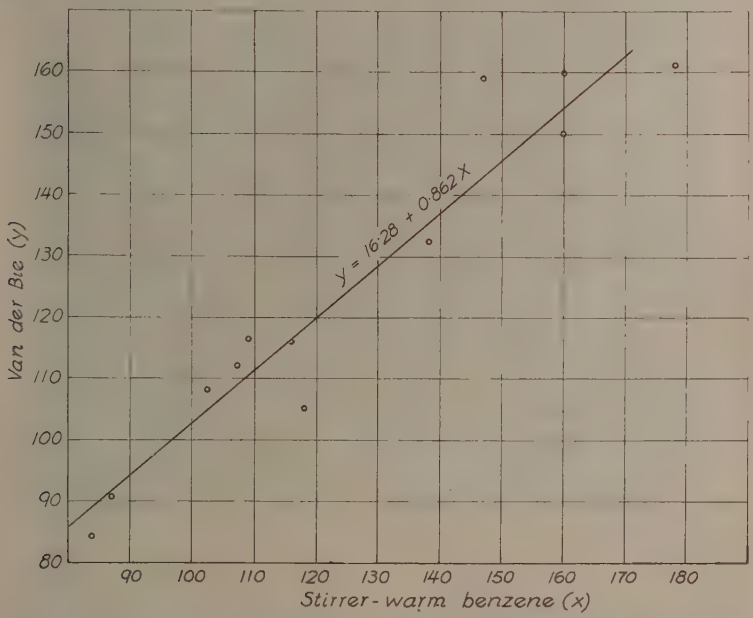


Figure 9. Relationship between amounts of rubber extracted from dried petiolar material by van der Bie's and warm benzene stirrer extraction methods. Values given as mg rubber per 5 gm of petiole material.

Using this method (x) a comparison with the standard van der Bie's method (y) gave a regression line (*Figure* 9) according to the equation: $y = 16.28 + 0.862x$, where b (slope of the regression) = 0.86 $\pm$ 0.08, and a = 16.3 $\pm$ 2.32. Consequently the slope of the line did not differ significantly from unity but constant a differs significantly from zero. Strictly therefore the warm benzene extraction method cannot be regarded as directly equivalent to van der Bie's method. The mutual relationship between both methods was however highly significant as also was the correlation coefficient (r_{yx} = 0.96).

It was also noted during this investigation that the readily extracted rubber content of dried petiole material decreased with time; hence it was desirable that all samples should be analysed as quickly as possible after drying. The rubber content of the petiole varied markedly with age; petioles taken from young leaves contained a smaller concentration of rubber than those taken from older leaves. It was therefore essential that comparisons should be carried out on petioles of a similar age.

The warm benzene stirrer extraction method has been extensively used for the estimation of the rubber content of stems and petioles; it has not, and was not intended to be used for the analysis of leaves or seed material.

THE DETERMINATION OF SUGARS PRESENT IN THE LAMINAE OF HEVEA BRASILIENSIS

The following procedure was found satisfactory for the preparation of leaf extracts suitable for both the chromatographic examination and quantitative estimation of the sugars present.

2 gm of fresh laminae were soxhlet extracted with 120 ml of 80% ethyl alcohol for two hours. The extract was evaporated under reduced pressure at 30-40°C to approximately 10 ml. This volume was partially clarified by the addition of 10 ml of 0.3 N barium hydroxide and 10 ml of 5% zinc sulphate solution, filtered and made up to a volume of 50 ml with water.

Twentyfive ml of this solution was concentrated under reduced pressure to a volume of 1 ml from which aliquots (usually 0.01 ml) were taken for the chromatographic examination.

Of the remaining 25 ml a 2.5 ml aliquot was taken, clarified by the addition of 5 ml of 0.3 N, barium hydroxide and 5 ml of 5% zinc sulphate solution, filtered and made up to a volume of 10 ml. This second clarification was necessary to ensure a perfectly colourless solution for the subsequent colorimetric estimation of the reducing and total sugars present.

The identity of the sugars present in clarified alcoholic leaf extracts was investigated by two dimensional paper chromatographic techniques. Butanol-ethanol-water (PARTRIDGE, 1946) and phenol-water (PARTRIDGE, 1948) were employed as the solvents and aniline phthallate (PARTRIDGE, 1949) and naphthoresorcinol in hydrochloric-phosphoric acid mixture (BRYSON and MITCHELL 1951) as the dipping agents.

Glucose, fructose and sucrose were always present in the leaf extracts examined. In addition relatively large amounts of inositol (both meso- and laevo-) occurred. It was possible to distinguish all these compounds on the same paper (*Figure* 10) by using a silver nitrate dipping agent (TREVELYAN, PROCTOR and HARRISON 1950).

Of the 10 ml volume prepared for the colorimetric estimation of the sugars, 2 ml were taken for the determination of the reducing sugars present (glucose and fructose). The procedure followed was identical with the technique described by SOMOGYI (1945) with the exception that after the addition of the copper

reagent the solution was heated for 30 minutes (not 10 min). For the estimation of the total sugars present (glucose, fructose and sucrose) a 1 ml aliquot was taken, hydrolysed by boiling for 30 minutes with 0.5 ml of 2 N sulphuric acid and neutralised by the addition of 0.5 ml of 2 N sodium hydroxide. The subsequent procedure was identical with that applied to the reducing sugars.

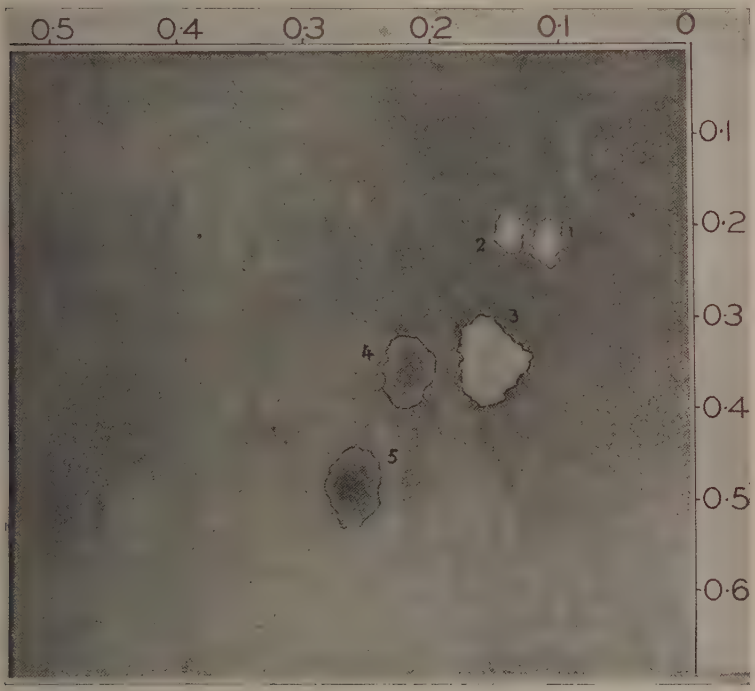


Figure 10. Two dimensional paper chromatogram. Solvents: phenol-water (horizontal R_F scale); butanol-ethanol-water (vertical R_F scale). Dipping reagent Ag NO_3.

Spot 1: meso inositol *Spot 2: laevo inositol*
Spot 3: sucrose *Spot 4: glucose*
Spot 5: fructose

The recovery values for known amounts of glucose added to the leaf extracts ranged from 90% to 110%; sucrose recovery values were generally less satisfactory and ranged from 85% to 120%.

The SOMOGYI (1945) method which was adopted for the colorimetric estimation of the sugars present in *Hevea brasiliensis* laminae was, although reliable, not specific and unable to distinguish the amount of glucose present from that of fructose. On the other hand the various quantitative procedures for eluting and measuring the amount of sugars in the chromatogram spots were often tedious, sensitive to error and although reliable

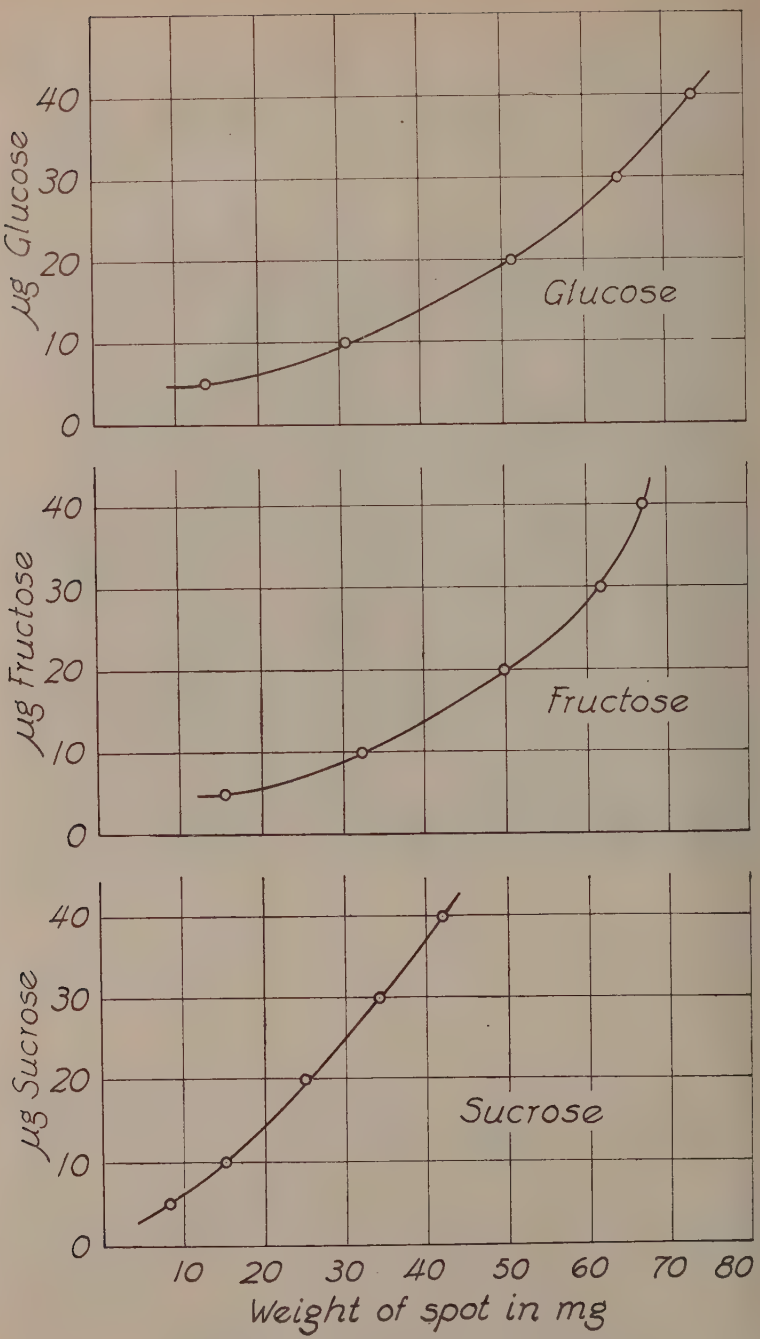


Figure 11. Standard curves obtained by plotting micrograms sugar 'spotted' against weight of cut out spot (in mg) using AgNo as the dipping agent.

when operated under rigorously controlled conditions, were unsuitable for routine estimations. These difficulties were overcome by using a simple paper chromatographic technique which has been developed and found successful. The new method has the advantages of simplicity, rapidity, and specificity, and is superseding the colorimetric procedure in this laboratory.

The method consisted in running single dimensional chromatograms of the alcoholic leaf extracts in a butanol-ethanol-water solvent (PARTRIDGE 1946) for 48 hours. The papers were then dipped in silver nitrate solution as described by TREVELYAN *et al* (1950). The spots were sufficiently separated to distinguish the three sugars and inositol which were present. When the papers were dried the spots were delineated with pencil, cut out, oven dried and weighed. The amount of sugar present in each spot was obtained by reference to a standard curve (relating weight of spot to μg sugar) prepared by spotting on different amounts of sugar mixtures, and running under the same conditions as the 'unknowns'. A range of 0-40 μg for each sugar was covered (*Figure* 11). The calibration curves were not exactly linear or reproducible; consequently fresh curves were prepared for each batch of estimations.

The method was applied only when the identity of the sugars present in the unknown had been definitely established by two dimensional chromatographic procedures. It was used for the estimation of sugars present in laminae borne on plants grown under different mineral status.

SUMMARY

An efficient sand culture technique has been put into operation for the production of *Hevea brasiliensis* plants of known nutritional status. Chemical methods for the estimation of the macro- and micro-nutrients in the laminae of *H. brasiliensis* have been investigated and satisfactory procedures established. The operation of a bioassay method for the estimation of traces of molybdenum and a colorimetric method of chlorophyll estimation are described.

A new rapid method suitable for the routine estimation of rubber in a large number of petiole samples has been evolved and put into use.

A new chromatographic procedure for the quantitative estimation of sugars in alcoholic leaf extracts is described. This method which is both specific and rapid was used for the estimation of each sugar present in the laminae of *H. brasiliensis*. The adaptation of the Somogyi method for reducing and total sugar estimation in leaf extracts is also described.

I thank Mr C.G. Akhurst and Mr G. Owen for facilitating the progress of the work described in this report and gratefully acknowledge the willing assistance of Mr K. Ratnasingam and others of the Soils Division in the experimental operations.

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February 1954

REFERENCES

A.O.A.C. (1945) *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists. George Banta Publishing Co, Wisconsin.*

BONNER, J. and ARREGUIN, B. (1949) The Biochemistry of Rubber Formation in the Guayule. I. Rubber Formation in Seedlings. *Arch. Biochem. 21*, 109

BRYSON, J.L. and MITCHELL, T.J. (1951) Improved Spraying Reagents for the Detection of Sugars on Paper Chromatograms. *Nature, Lond. 167*, 864

BURGER, V.L. DONALDSON, W.E. and BATY, J.A. (1943) A Direct Determination of Rubber Hydrocarbon. Chromic Acid Oxidation Method. *Rubb. Chem. Technol. 16*, 660

CHIBNALL, A.C. REES, M.W. and WILLIAMS, E.F. (1943) The Total Nitrogen Content of Egg Albumin and other Proteins. *Biochem. J. 37*, 354

COMAR, C.L. (1942) Analysis of Plant Extracts for Chlorophylls a and b using a Commercial Spectrophotometer. *Industr. Engng Chem. (Anal.) 14*, 877

COMAR, C.L. BENNE, E.J. and BUTEYN, E.K. (1943) Calibration of a Photoelectric Colorimeter for the Determination of Chlorophyll. *Industr. Engng Chem. (Anal.) 15* 524

COMAR, C.L. and ZSCHEILE, F.P. (1942) Analysis of Plant Extracts for Chlorophylls a and b by a Photoelectric Spectrophotometric Method. *Plant Physiol. 17*, 198

CORNFIELD, A.H. and POLLARD, A.G. (1950) The use of Tetramethyl Diaminodiphenyl Methane for the Determination of Small Amounts of Manganese in Plant Material and Soil Extracts. *J. Sci. Fd. Agric. 1*, 107

DRAKE, G.W. (1950) an unpublished account of the R.R.I. method of copper determination

DROSDOFF, M. and NEARPASS, D.C. (1948) Quantitative Micro-Determination of Magnesium in Plant Tissue and Soil Extracts. *Analyt. Chem. 20*, 673

FORSTER, W.A. (1949) an unpublished account of potassium estimation method

HATCHER, J.H. and WILCOX, L.V. (1950) Colorimetric Determination of Boron Using Carmine. *Indstr. Engng Chem. (Anal.) 22*, 567

HEWITT, E.J. (1952) Sand and Water Culture Methods used in the Study of Plant Nutrition. Communication 22 *Tech. Commun. Bur. Hort., E. Malling*

HEWITT, E.J. and HALLAS, D.G. (1951) The use of *Aspergillus Niger* (van Tiehg) M Strain as a Test Organism in the Study of Molybdenum as a Plant Nutrient. *Plant & Soil 3* 366

HEINEN, E.J. and BENNE, E.J. (1951) Report on Zinc in Plants. *J. Ass. off. agric. Chem. Wash. 34*, 692

HUNTER, J.G. (1950) An Absorptiometric Method for the Determination of Magnesium. *Analyst 75*, 91

MASON, A.C. (1951) The Estimation of Iron in Plant Material. *Rep. E. Malling Res. Sta.* (1950) 124

MEEKS, J.W. CROOK, R.V. PARDO, C.E. and CLARK, F.E. (1953) Determining Rubber in Rubber Bearing Plants. *Analyt. Chem. 25*, 1535

PARTRIDGE, S.M. (1946) Application of the Paper Partition Chromatogram to the Qualitative Analysis of Reducing Sugars. *Nature. Lond. 158*, 270

PARTRIDGE, S.M. (1948) Filter-Paper Partition Chromatography of Sugars. 1. General Description and Application to the Qualitative Analysis of Sugars in Apple Juice, Egg White and Foetal Blood of Sheep. *Biochem. J. 42*, 238

PARTRIDGE, S.M. (1949) Aniline Hydrogen Phthalate as a Spraying Agent for Chromatography of Sugars. *Nature, Lond. 164*, 443

PICKARD, J.A. (1952) an unpublished method of the City Analyst Bristol, for iron estimation using thioglycollic Acid

RICHARDS, M.B. (1930) The Colorimetric Determination of Manganese in Biological Material. *Analyst 55*, 554

RICHES, J.P.R. (1948) unpublished communication on Traub's turbidometric method for estimation of rubber

SANDELL, E.B. (1944) *Colorimetric Determination of Traces of Metals.* Interscience Publishers, N.Y.

SLATTERY, M.C. and TYSDALL, H.M. (1950) unpublished communication on a rapid rubber assay method in guayule shrub by means of the Waring Blendor

SOMOGYI, M. (1945) The Determination of Reducing Sugars. *J. biol. Chem. 160*, 61

SPENCE, D. and CALDWELL, M.L. (1933) Determination of Rubber in Rubber Bearing Plants. *Indstr. Engng Chem. (Anal.) 5*, 371

STREET, H.E. KENYON, A.E. and WATSON, G.M. (1946) The Assimilation of Ammonium and Nitrate Nitrogen by Detached Potato Sprouts. *Ann. appl. Biol. 33*, 369

TRAUB, H.P. (1946) Rapid Photometric Methods for Determining Rubber and Resins in Guayule Tissue and Rubber in Crude-Rubber Products. *United States Department of Agriculture Washington, D.C. Tech. Bul.* 920

TREVELYAN, W.E. PROCTOR, D.P. and HARRISON, J.S. (1950) Detection of Sugars on Paper Chromatograms *Nature, Lond. 166*, 444

VAN DER BIE, G.J. (1946) Determination of the Rubber Content of Hevea Leaves. *India Rubb. J. 111*, 417

WILLARD, H.H. and GREATHOUSE, L.H. (1917) Colorimetric Determination of Manganese by Oxidation with Periodate. *J. Amer. chem. Soc. 39*, 2366

WILLITS, C.O. SWAIN, M.L. and OGG, C.L. (1946) Determination of Rubber Hydrocarbon by a Gravimetric Rubber Bromide Method. *Indstr. Engng Chem. (Anal.) 18*, 439

Printed by
Charles Grenier & Son, Ltd.
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